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Change in Chemical Composition of Lipids Accumulated in Atheromas of Rabbits Following Photodynamic Therapy

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Background and Objective: **Mono-L-aspartyl chlorin e6 (NPe6) has been used in the photodynamic therapy of neoplasms. This substance has been shown to accumulate in atheroma of rabbits. We evaluated the change in the lipid components of atheromas after photodynamic therapy with NPe6 using Fourier transform infrared (FTIR) microspectroscopy.**
Study Design/Materials and Methods: **Rabbits were fed a cholesterol-rich diet for 20 weeks. Six hours after they were administered NPe6 (2mg/kg), the atheroma present on the abdominal aorta was irradiated with a diode laser with 100J/cm^2 or 200J/cm^2 of the tissue fluence. Tissue samples were prepared for FTIR and histological analysis.**
Results: **Specimens of atheroma from the untreated animals appeared as fatty streaks whose infrared spectra exhibited characteristic peaks at 1,738^{-1} cm^{-1}, 1,468 cm^{-1}, 1,380 cm^{-1}, and 1,174 cm^{-1}, indicating the accumulation of cholesterol ester. Seven days after photodynamic therapy, FTIR microspectroscopic analysis showed a marked decrease in the peak intensity in the treated atheroma related to the =C=O ester bond at 1738 cm^{-1} with no concomitant increase in the intensity of the peaks related to free cholesterol.**
Conclusion: **Findings suggest a dissociation of ester bonds and the depletion of cholesterol esters in the atheromas after photodynamic therapy with NPe6. The lipids accumulated in the atheroma were perhaps decreased or destroyed following such treatment. Lasers Surg. Med. 21:287–293, 1997.**

Key words: atherosclerosis; cholesterol ester; diode laser; Fourier transformation; infrared microspectroscopy; mono-L-aspartyl chlorin e6; photosensitizer

INTRODUCTION

Photodynamic therapy is considered useful in treating some forms of cancer in humans [1–5]. Second-generation photosensitizers such as mono-L-aspartyl chlorin e6 (NPe6) and benzoporphyrin derivatives have been developed to improve efficacy and to reduce the incidence of side effects associated with such therapy [5,6]. We previously reported that NPe6 accumulates in atherosclerotic plaques as well as in malignant tumors [7–10]. Six hours after the administration of NPe6 to rabbits, this substance was localized in

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atheroma [8–10]. We hypothesized that photodynamic therapy using NPe6 might be beneficial in treating atherosclerotic lesions.

Fourier transform infrared (FTIR) spectroscopy has been applied to a cardiovascular field to characterize the tissue type using the conventional transmission method [11,12]. Recently, the use of FTIR spectroscopy using an infrared microscope having a high signal-to-noise ratio has made it possible to determine the infrared spectra of tissue materials [13,14] and to analyze the chemical components of tissue slices 10 μm thick [15].

Our objective was to evaluate the effects of photodynamic therapy on the chemical composition of the lipids present in the atheromatous plaques of rabbits using FTIR microspectroscopy.

MATERIALS AND METHODS

We evaluated 21 female New Zealand white rabbits. Fourteen of them were fed an atherogenic diet that contained 0.5% cholesterol for 20 weeks. After 4 weeks on this diet, their serum concentration of cholesterol consistently exceeded 1,200 mg/dl. Seven control rabbits were fed a normal diet and had a serum cholesterol concentration below 100 mg/dl at the initiation of the experiment.

The animal experiments complied with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85–23, revised 1985).

The photosensitizer, NPe6 (Meiji Seika Kaisha, Tokyo, Japan), is a dark blue-green, water-soluble compound. The absorption spectrum of NPe6 demonstrated the main absorption peaks at 400 nm and 664 nm.

NPe6, 2 mg/kg, was injected into the ear vein of 10 atherosclerotic and 4 control rabbits. Six hours after NPe6 administration, the rabbits were anesthetized with pentobarbital sodium (30 mg/kg i.v.). The abdomen was opened under sterile conditions and the abdominal aorta was exposed. Atherosclerotic lesions of the abdominal aorta were grossly observed as white plaques. A diode laser, wavelength 664 nm, which was generated with a laser device (Matsushita Industrial Equipment Co. Osaka, Japan), was applied to the atherosclerotic plaque via the adventitia of the abdominal aorta. A laser beam with a wavelength of 664 nm was transmitted through a quartz fiberoptic wave guide from a diode laser device. The tip of the guide was placed 0.5 cm above the adventitia of the abdominal aorta. An area 1 cm in diameter was irradiated 0.5 cm from the tip. The area selected for laser irradiation was just below the celiac artery or the renal artery bifurcation, at which the atherosclerotic plaque was observed. Thus, the irradiated portion of the aorta could be recognized seven days after treatment. The tissue fluence was 100J/cm^2 (n = 6) or 200J/cm^2 (n = 4) at a power of 40 mW/cm^2. Four control rabbits were similarly exposed to photodynamic therapy, but with 100J/cm^2. The four remaining atherosclerotic rabbits were exposed to a diode laser (100J/cm^2) without the injection of NPe6. After treatment, all atherosclerotic rabbits received a normal diet.

Seven days after the photodynamic therapy or laser irradiation given alone, 18 rabbits were killed by the injection of an overdose of pentobarbital sodium. The abdominal aorta was quickly excised and sliced in cross sections 10 μm thick with a freezing microtome. Each slice was immediately placed on a glass slide made of calcium fluoride (CaF_2) (Oken Co., Tokyo, Japan) and air dried at room temperature. Tissue samples from the normal aortas of the three remaining, untreated, control rabbits were similarly prepared.

A microscopy FTIR spectrophotometer (Micro FT/IR-100) (JEOL, Tokyo, Japan) was used. Although tissue samples usually contain some water that influences the spectra [16,17], analysis of a thin layer of tissue sample was feasible in this system because we subtracted the spectra of water, as well as that of CO_2 that was present in the tissues from the spectrum of the sample. Lipids in a tissue sample had a negligible polarizing effect on the infrared absorption spectrum (data not shown). An area 100 μm^2 was analyzed with the microscopy FTIR spectrophotometer. Several different points were measured in each tissue sample. The infrared absorption spectra of pure cholesterol, cholesteryl linoleate, and cholesteryl oleate (Sigma, St Louis, MO), were determined for comparison with those of tissue samples. The measured spectra were converted to absorption spectra by the data processor on the spectrophotometer. All spectra were obtained at a resolution of 4 cm^{-1}, range 4,300–500 cm^{-1}, with 100–200 scans.

Specimens adjacent to those prepared for FTIR analysis were stained with hematoxylin and eosin for microscopic analysis to confirm the presence of atheromatous plaques and were stained with Sudan IV to identify the presence of lipids.

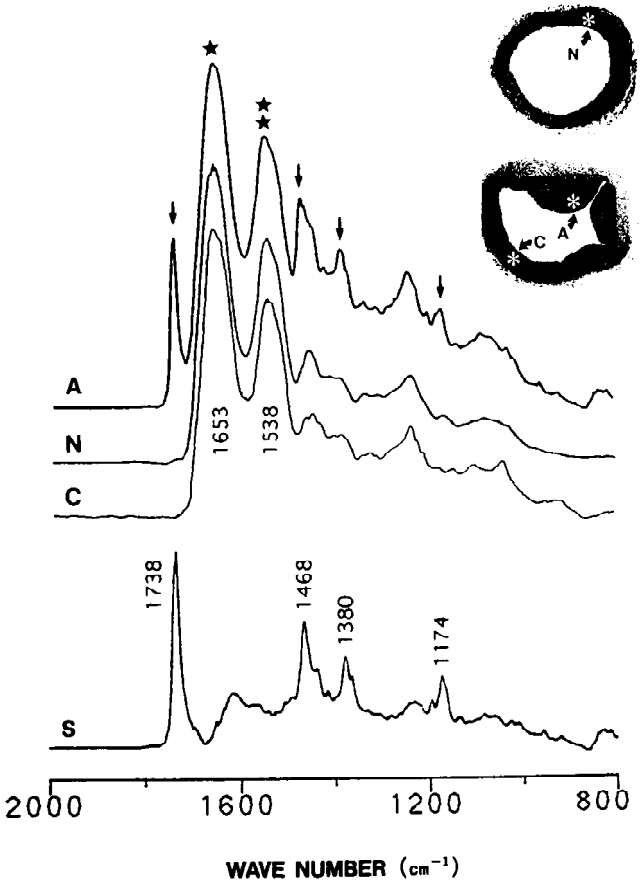

Fig. 1. Infrared absorption spectra obtained with a sample of atheroma from the abdominal aorta of an atherosclerotic rabbit **(A)** and that from normal wall without atheroma in the same sample **(C).** We observed major differences between the spectrum (C) and the spectrum obtained with a specimen of normal aorta from a control (non-atherosclerotic) rabbit **(N).** Absorption peaks for amide I (*) and amide II (**) were observed in the spectra of these specimens. Differences between spectra A and N were observed at the peaks indicated by arrows. Differences became more evident when N was subtracted from A **(S).**

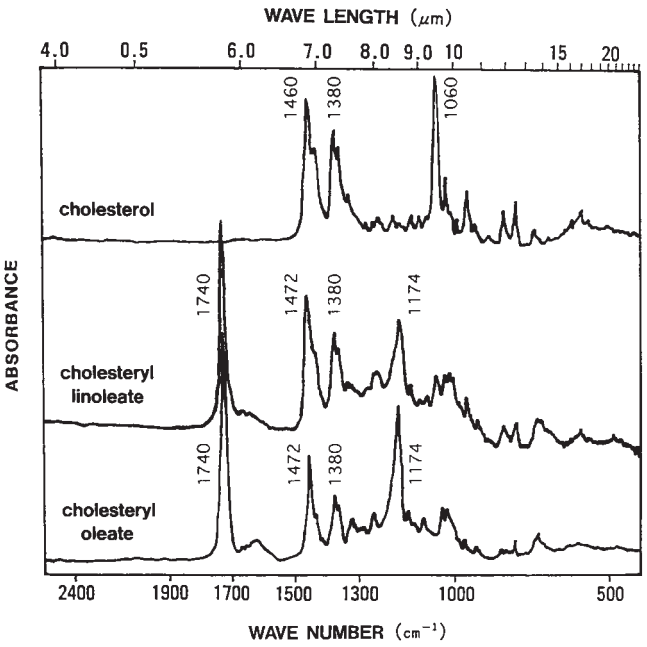

Fig. 2. Infrared spectra of pure cholesterol, cholesteryl linoleate, and cholesteryl oleate.

RESULTS

The infrared absorption spectra measured in a sample of aortic tissue revealed numerous peaks, of which 1,653 cm^{-1} and 1,538 cm^{-1} were characteristic of both the normal (Fig. 1N) and the atheromatous aorta of the rabbit (Fig. 1A). These two peaks corresponded to the well-known amide I and amide II absorption peaks that are consistently seen in the absorption spectra of proteins.

To demonstrate the characteristics of the infrared absorption spectra of the atheroma, the spectrum of the normal aorta was subtracted from that of the atheroma in the abdominal aortas of rabbits fed a cholesterol-rich diet for 20 weeks. The four specific peaks observed (at 1,738 cm^{-1}, 1,468 cm^{-1}, 1,380 cm^{-1}, and 1,174 cm^{-1}) were characteristic of the atheroma (Fig. 1S).

These peaks corresponded to the specific absorption peaks of the infrared spectra of specific pure chemicals (Fig. 2). Free cholesterol exhibited three high peaks (at 1,460 cm^{-1}, 1,380 cm^{-1}, and 1060 cm^{-1}). The peak at 1,460 cm^{-1} was related to a scissoring vibration of $-CH_2-$ in the cholesterol skeleton [18]. With esterification, this vibration band shifted to 1,472 cm^{-1} for both the oleate and the linoleate of cholesterol. The peak at 1,468 cm^{-1} seen with the atheroma (Fig. 1 A,S) was consistent with that observed at 1,472 cm^{-1} for the cholesterol esters. The 1,060 cm^{-1} peak was also related to the cholesterol skeleton, which was assigned the mode of -C-C- stretching vibration of the cholesterol skeleton. This peak moved to 1,174 cm^{-1} in the esterified cholesterol. The 1,174 cm^{-1} peak was small, but clearly present in atheroma. The peak at 1,380 cm^{-1} reflected the symmetrical deformation of $-CH_3$ groups were observed at the same position in the spectra of the atheroma as well as the cholesterol esters. A highly characteristic absorption peak for atheroma was present at 1,738 cm^{-1}. This corresponded to the 1,740 cm^{-1} peak of the cholesterol esters and was assigned the stretch vibration of $=C=O$. This peak was

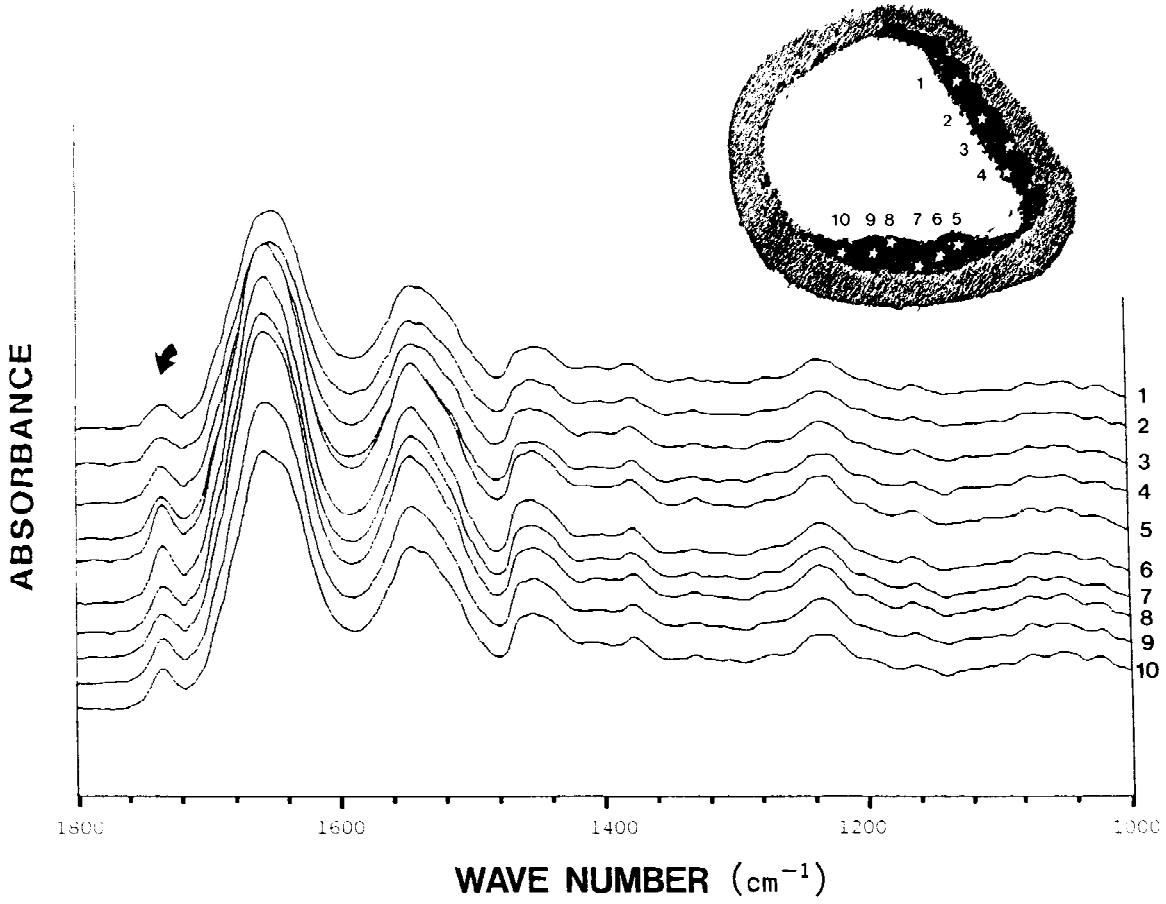

Fig. 3. Infrared absorption spectra obtained from 10 points (asterisks) from a position just under the endothelium to one near the media, in atheroma that were induced in the abdominal aorta by the prolonged administration of a cholesterol-rich diet. Each point was measured by Fourier transform infrared microspectroscopy. A characteristic peak at 1,738 cm^{-1} (arrow) was observed in each spectrum.

not observed in either the specimens of free cholesterol or in the specimens of the normal aorta.

The infrared spectrum measured at various points in the *untreated atheroma* exhibited a similar pattern with a characteristic peak seen at 1,738 cm^{-1}, although the intensity of this peak varied (Fig. 3).

Specimens of the abdominal aorta obtained 7 days after the completion of photodynamic therapy showed a reduction in cellular content (Fig. 4D) and a poor staining with Sudan IV in the treated atheromas. FTIR microspectroscopic analysis showed no peak at 1,738 cm^{-1} in any atherosclerotic plaque following photodynamic therapy with 100J/cm^2 (Fig. 5b–f). The infrared spectra of atheromas in the groups subjected to 200J/cm^2 exhibited similar patterns with an absence of the 1,738 cm^{-1} peak. However, this peak persisted in the specimens of atheromas that were treated with laser alone (Fig. 5a). Photodynamic therapy of four controls with a normal abdominal aorta showed no marked change in infrared spectra.

DISCUSSION

We demonstrated that the infrared spectra of the atheroma of untreated rabbits showed four characteristic peaks. Such peaks became more evident after subtracting the spectrum of the normal aorta from that of the atheroma. These peaks were consistent with those of cholesteryl esters [18]. The esterified cholesterol is a main lipid accumulated in the atheroma in humans and in rabbits fed a cholesterol-rich diet. Thus, the in situ detection of cholesterol esters in the aortic atherosclerotic plaques of rabbits was feasible by FTIR microspectroscopy.

Cholesteryl oleate and cholesteryl linoleate each showed a strong absorption peak at 1,740

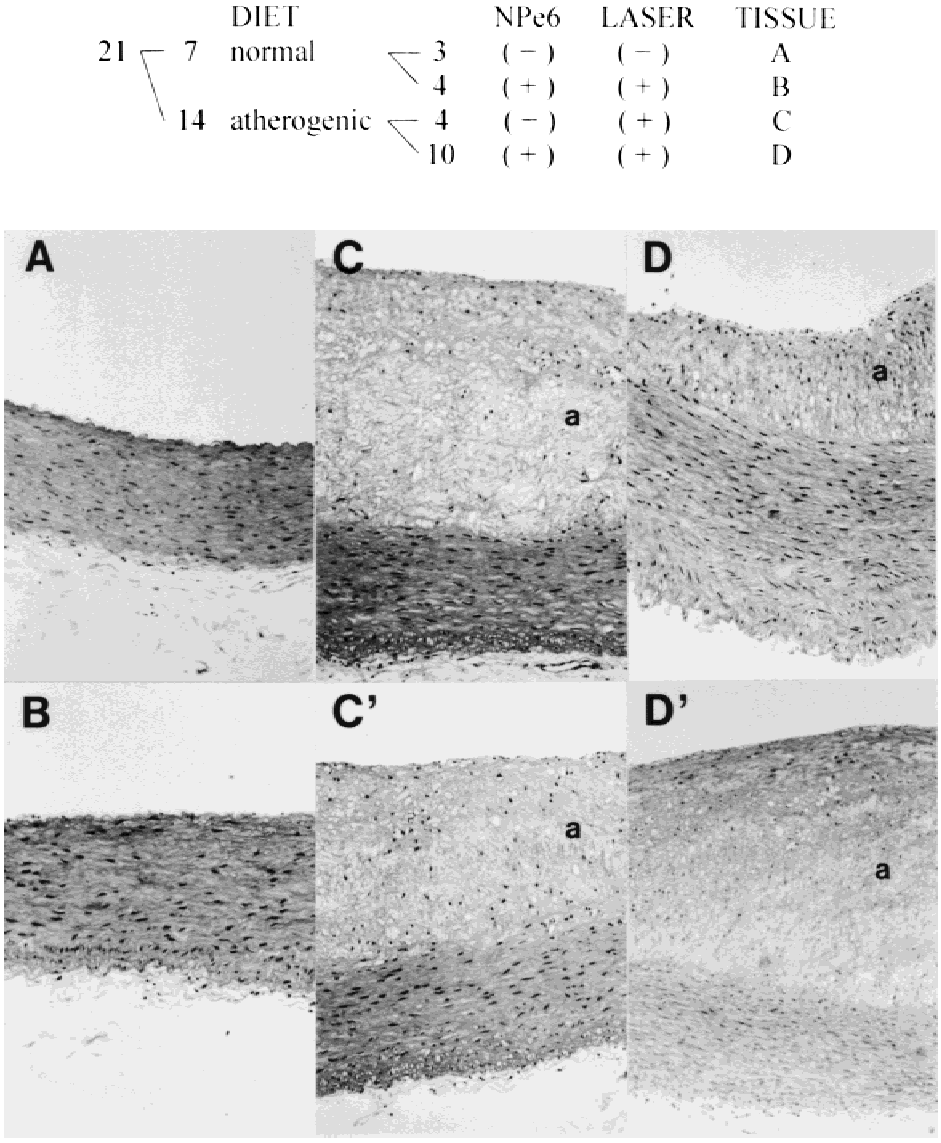

Fig. 4. Light micrographs of tissue samples prepared from rabbits administered each treatment (upper panel) (hematoxylin and eosin). **A** is a cross section of the abdominal aorta of an untreated, control (non-atherosclerotic) rabbit. The treated aorta in a control rabbit with photodynamic therapy **(B),** or in an atherosclerotic rabbit with laser irradiation alone **(C)** did not show any histological differences in the endothelium to media as compared with A or **C',** respectively. Micrograph C' was prepared from the untreated aorta of the same rabbit shown in C. Atheroma **(a)** in a specimen obtained 7 days after photodynamic therapy **(D)** shows a reduction in cellular content as compared with an atheroma that was not irradiated by laser in the same rabbit **(D').**

cm^{-1}, which is not seen with free cholesterol or fatty acids [18]. This 1,740 cm^{-1} peak corresponded to a peak at 1,738 cm^{-1} in the original, nonsubtracted spectrum of the atheroma. The 1,738 cm^{-1} peak was not seen in any specimen of the normal rabbit abdominal aorta. Peaks at 1,740 cm^{-1} and 1,738 cm^{-1} reflected the stretching vibrational absorption of the $=C=O$ ester bond and is thus characteristic of esterified cholesterols [18]. We previously demonstrated that the intensity of the peak at 1,738 cm^{-1} in the atherosclerotic lesion increased progressively in accordance with the duration of the atherogenic diet [13]. Other peaks between 1,500 to 800 cm^{-1} characteristic of the atheroma were not clearly identified, as numerous other peaks were present. Thus a specific peak at 1,738 cm^{-1} in the spectrum of the atheromas was considered to be a marker for the presence of cholesterol esters in the tissue.

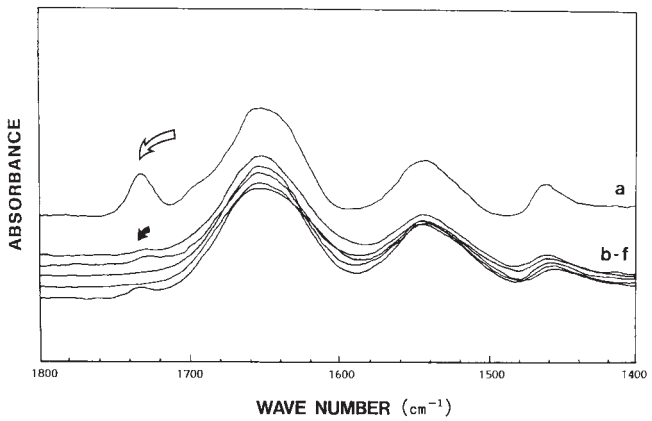

Fig. 5. Infrared absorption spectra obtained from atheroma 7 days after diode laser irradiation (100J/cm^2) with **(b–f)** or without **(a)** pretreatment with NPe6. The peak characteristic of atheroma, which was seen at 1,738 cm^{-1}, was absent (solid arrow) from the five spectra measured in the atherosclerotic plaques of tissue samples that were prepared 7 days after the photodynamic therapy. However, this peak persisted (open arrow) in the spectrum of an atheroma in an animal that was treated only with a diode laser (100J/cm^2). The light micrographs that correspond to the tissue samples in which spectra (a) and (b) were measured are shown in Figure 4C, D, respectively.

A disappearance of the peak at 1,738 cm^{-1} was the most marked change in the spectrum following photodynamic therapy from the treated vs. the untreated atheroma. This suggests a dissociation of the $=C=O$ double bond in the chemical structure of the cholesterol esters. The absence of a concomitant increase in the intensity of the peaks related to free (unesterified) cholesterol in the infrared spectra of specimens obtained from animals with treated atheroma suggests a depletion of the cholesterol esters from plaque in such treated animals. The lipids that accumulated in atheroma as esterified cholesterols during the prolonged feeding of a diet high in cholesterol may have been destroyed following photodynamic therapy with NPe6.

Irradiation of the atheromas with a diode laser without NPe6 produced no marked change in spectra. Laser irradiation of control rabbits that were pretreated with NPe6 did not markedly change the spectra of the normal abdominal aorta that was free of atheroma. However, a marked decrease in the intensity of the 1738 cm^{-1} peak was demonstrated in atheroma after the administration of photodynamic therapy to the atherosclerotic rabbits. We therefore consider that photodynamic therapy with NPe6 had a high specificity for atheroma, which was dependent on the accumulation of NPe6 in the atherosclerotic plaques [8–10].

The present study is the first to describe the breakdown of the esterified cholesterol accumulated in the atheroma of rabbits following the administration of photodynamic therapy with evidence provided by FTIR microspectroscopy. The combination of a diode laser and NPe6 is considered to be useful in stabilizing or reducing the size of atheromas. Further investigation is needed before photodynamic therapy can be used for treating atherosclerotic lesions of humans, which may not be the simple fatty streaks seen in rabbits, but, rather, fibrous, more complex, calcified plaques.

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